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(54) Title: A NEW BACTERIAL TOXIN FOR USE AS A PLANT GROWTH REGULATOR AND HERBICIDE

(57) Abstract

A herbicidal composition, tabtoxinine-β-lactam, has been isolated from a plant pathogenic bacterium, *Pseudomonas syringae* (ATCC #55090). The herbicidal activity can be isolated from culture medium in which this bacterium has grown. Topical application to the foliage of plants causes rapid water soaking and chlorosis. The toxin is transported throughout the weed from the site of topical application of the herbicidal composition via vascular transport, and results in commercial levels of weed control. Combinations of the toxin with chemical herbicides produce accelerated and enhanced injury to weeds.

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Description
A New Bacterial Toxin For Use As A Plant Growth Regulator And Herbicide

This application is a continuation-in-part of application Serial No. 08/069,007, filed May 28, 1993, now abandoned, and of application Serial No. 08/069,008, filed May 28, 1993, now abandoned, and of application Serial No. 08/142,943, filed October 29, 1993.

Field of the Invention

The present invention relates to a herbicidal composition produced by bacteria, methods for its production, and its use for controlling the growth of plants.

Description of Related Art

Widespread use of synthetic chemical herbicides has created concern about possible adverse ecological effects caused by the large quantities of chemical agents that are being introduced into the environment. There are fears that these chemicals pose unknown or unexpected risks to human health and the environment. Special concern has been directed to health effects on agricultural workers, who may be exposed to high levels of chemical herbicides. It has been necessary to withdraw from the market or severely restrict the use of some chemical herbicides after adverse environmental or health effects became evident. Disposal of containers for chemical herbicides is heavily regulated, and proper disposal of these containers can be inconvenient and expensive for farmers. Disposal sites for these containers are limited and illegal container disposal is a growing concern.

Thus, there exists a need in the art for improved and safer compositions and methods for controlling a broad range of weed species to replace the synthetic herbicidal chemicals currently used for this purpose.

Some microorganisms produce substances that are toxic to plants. One strategy for developing less environmentally harmful herbicides is to identify and isolate phytotoxins produced by microorganisms. If such toxins can be used as herbicides, then these naturally-occurring agents may have fewer deleterious environmental effects than the synthetic chemicals currently used for weed control. Effort has been directed at identifying and isolating phytotoxins made by microorganisms for possible use as herbicides. Such efforts have usually involved attempts to identify and isolate phytotoxins from culture media in which bacteria or fungi have grown.

The isolation and identification of herbicidal compounds made by microorganisms faces numerous obstacles. The process of identifying, purifying, and isolating such compounds is lengthy and expensive. Media in which microorganisms have grown (conditioned media) are highly complex mixtures and the active compounds with herbicidal activity may be present in extremely low concentrations. If the active compounds are labile, or if herbicidal actions are due to synergistic interactions of multiple components, then the activity may be lost during purification. Even where an active compound is identified, the production of commercially useful quantities of such compounds by industrial fermentation and purification or by chemical synthesis may require considerable additional effort.

Perhaps because of the difficulties and expense of isolating microbially-derived herbicidal compounds, only a few natural products made by microorganisms have been developed to the stage where they could be used as commercial herbicides. One example is bialaphos, L-2-amino-4-[(hydroxy)(methyl)phosphinoyl]-butyl-L-alanyl-L-alanine, which is sold under the trade name HERBIACE®. A chemically synthesized derivative of bialaphos, glufosinate (IGNITE®, BASTA® or FINALE®), is also used as a herbicide. Efforts to develop herbicides from microbial phytotoxins are described in Duke, S.O., "Microbially produced phytotoxins as

herbicides -- a perspective," in Putnam, A.R. and Tang, C.-S. (Eds.) The Science Of Allelopathy, New York, John Wiley & Sons, 1986, p.287.

Pathovars of Pseudomonas syringae can infect plants, and some phytotoxins produced by these bacteria have been identified. For example, U.S. Patent No. 4,874,706 to Durbin et al. discloses the isolation of a plant toxin (tagetitoxin) produced by Pseudomonas syringae pv. tagetis. Tagetitoxin has not been developed as a commercial herbicide. Another toxin, tabtoxin, is produced by certain pathovars of Pseudomonas syringae including Pseudomonas syringae pv. tabaci, Pseudomonas syringae pv. coronafaciens and Pseudomonas syringae pv. glycine. While various phytotoxins produced by microorganisms have been isolated and their mechanisms of actions studied, they have not been successfully developed as commercial herbicides.

U.S. Patent Application Serial No. 08/050,301, filed May 17, 1993, incorporated herein by reference, the disclosure of which has been published on May 29, 1992, as PCT application publication No. WO 92/08357, discloses that media in which bacteria have grown can facilitate the injury to plants caused by low or moderate levels of chemical herbicides. For example, medium conditioned by the bacterium Pseudomonas syringae ATCC #55090 has little effect when sprayed on a variety of weeds. However, when this medium is applied in combination with relatively low levels of chemical herbicides, the injury to the weeds is greater than is caused by the herbicide alone. As shown in WO 92/08357, combining a herbicide with a conditioned culture medium provides a convenient and inexpensive method to enhance the activity of the herbicide without any need to identify or purify the active components of the conditioned medium.

One possible way that a conditioned medium may enhance the effects of a chemical herbicide is that the medium may contain a phytotoxin which is present in too low a concentration to cause measurable injury when applied alone but which enhances the action of the chemical herbicide.

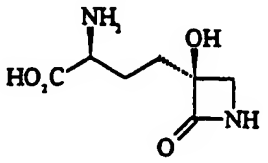
Thus, adding low levels of a known herbicide to a medium in which bacteria have grown can be used to assay for phytotoxins excreted by bacteria into culture medium, even though the toxin may be present in amounts insufficient to cause injury when the conditioned medium alone is applied to plants.

Using this approach, we have identified a herbicidal toxin produced by a bacterium, Pseudomonas syringae ATCC #55090. When this toxin is applied to the foliage of plants, it causes rapid water soaking and chlorosis. The toxin moves through the plants via the vascular system from the site of application to target sites, thus killing the entire weed plant and resulting in commercial levels of control of unwanted target vegetation.

The active herbicidal component of the toxin produced by Pseudomonas syringae ATCC #55090 is tabtoxinine-β-lactam (2-amino-4-(3-hydroxy-2-oxoazacyclobutan-3-yl)-butanoic acid; "TBL") (1). As used herein, "toxin" or "phytotoxin" refers to TBL, both in its purified and unpurified forms. The term "toxin" or "phytotoxin" also refers to the derivatives and salts of TBL.

Tabtoxin (2) is an unusual dipeptide containing a tabtoxinine-β-lactam residue linked to the amino group of threonine or serine. W.W. Stewart, Isolation and proof of structure of wildfire toxin, Nature 229:174-178 (1971). Tabtoxin is produced by pathovars of Pseudomonas syringae, including Pseudomonas syringae pv. tabaci, Pseudomonas syringae pv. coronafaciens and Pseudomonas syringae pv.

glycine. Tabtoxin readily translactamizes to the biologically inactive but more stable δ-lactam (3).



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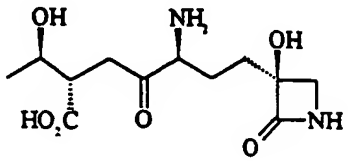

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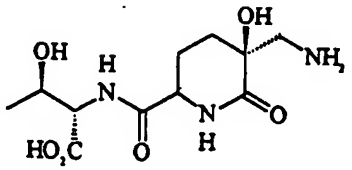

(3)

Tabtoxin was historically thought to be the active toxin, but tabtoxin was later shown to be a precursor that undergoes hydrolysis to yield the biologically active form, TBL. Uchytil, T.F. and Durbin, R.D., "Hydrolysis of Tabtoxin by Plant and Bacterial Enzymes," Experientia 36:301-302 (1980). The NMR spectrum of tabtoxin is described by W.W. Stewart, "Isolation and Proof of Structure of Wildfire Toxin," Nature, 229:174-178 (1971).

Methods for the isolation of tabtoxin produced by bacterial fermentation are known to those of skill in the art. See, e.g., Wooley, D.W., Pringle, R.B. and Braun, A.C., "Isolation of the Phytopathogenic Toxin of Pseudomonas tabaci, an Antagonist of Methionine," J. Biol. Chem., 197:409-417 (1952).

Chemical syntheses of tabtoxin and tabtoxinine-β-lactam (TBL) are known to the art and have been described in the literature. J. E. Baldwin, et al., "Stereospecific Synthesis of Tabtoxin," Tetrahedron, 40(19):3695-3700 (1984); J. Baldwin et al., "Synthetic Studies on Tabtoxin. Synthesis of a Naturally Occurring Inhibitor of Glutamine Synthetase, Tabtoxinine-β-lactam, and Analogues," Tetrahedron 42(12):3097-3110 (1986).

Tabtoxinine-β-lactam has been reported to inhibit glutamine synthetase. Thomas et al., "Inhibition of

Glutamine Synthetase from Pea by Tabtoxinine-β-lactam," Plant Physiol. 71:912-915 (1983). The production and regulation of tabtoxin production has been studied at the genetic level. See T.G. Kinscherf, et al., "Cloning and Expression of Tabtoxin Biosynthetic Region From Pseudomonas syringae," J. Bacteriol. 173:4124-4132 (1991); T.M. Barta, et al., "Regulation of Tabtoxin Produced by the lemA Gene in Pseudomonas syringae," J. Bacteriol. 174:3021-3029 (1992).

Although tabtoxinine-β-lactam has been isolated previously and its mechanism of action studied, it has never been successfully developed as a commercial herbicide. It is highly surprising and unexpected that tabtoxinine-β-lactam can be used as a commercial herbicide. Indeed, there are no reports in which tabtoxinine-β-lactam was topically applied on the surface of weeds or to soil. In addition, there have been no reports that tabtoxinine-β-lactam topically applied to one leaf of a plant will move to other leaves and kill the entire plant.

Summary Of The Invention

We report here for the first time that tabtoxinine-β-lactam (TBL) can be used to control weeds when topically applied to the epidermal surface of the weed or applied to soil in which the weed is growing. The herbicidal activity of TBL is not dependent upon internal delivery via infiltration or injection as prior art has taught. Topically applied TBL is able to move throughout the plant via the vascular system, an unexpected result given the prior art.

Accordingly, the present invention is directed to compositions and methods that substantially obviate one or more of the problems due to limitations and disadvantages of the related art. It has now been discovered that TBL can be topically delivered to weeds or soil via standard commercial application methods to reproducibly achieve commercial levels of weed control in the field. Topically applied TBL moves rapidly across the plant epidermis and inside plants it moves

from the site of application throughout the plant, resulting in highly effective control of unwanted target vegetation.

The present invention comprises methods for the control of weeds by topically applying a herbicidal composition comprising tabtoxinine-β-lactam to the weeds as a plant growth regulator and herbicide. The terms "tabtoxinine-β-lactam" and "toxin" as used herein encompasses not only tabtoxinine-β-lactam but also its herbicidal salts, analogues and derivatives, including precursor compounds such as tabtoxin.

The present invention further comprises methods for the production of tabtoxinine-β-lactam produced by fermentation by a bacterium, Pseudomonas syringae ATCC #55090.

Another embodiment of the invention comprises employing tabtoxinine-β-lactam produced by Pseudomonas syringae ATCC #55090 for the control of weeds.

The present invention also comprises a naturally occurring herbicidal composition produced by Pseudomonas syringae ATCC #55090 that is useful as a broad spectrum herbicide.

The present invention comprises methods for the production of this herbicidal composition by fermentation of the bacterium Pseudomonas syringae ATCC #55090.

Another embodiment of the present invention comprises a strain of Pseudomonas syringae ATCC #55090.

Another embodiment of the invention comprises methods for the control of weeds by the topical application of tabtoxinine-β-lactam in combination with chemical herbicides.

Another embodiment of the invention comprises herbicidal compositions comprising mixtures of tabtoxinine-β-lactam and chemical herbicides.

Another embodiment of the invention comprises compositions comprising tabtoxinine-β-lactam for use as harvest aids, and the use of such compositions as a harvest aid to defoliate crops such as potatoes, peanuts and cotton.

Another embodiment of the invention comprises a method of controlling the growth of weeds by topically applying tabtoxinine-β-lactam to the foliage or roots of emergent weeds.

Another embodiment of the invention comprises a method for controlling weeds to prevent or inhibit weed growth which comprises applying tabtoxinine-β-lactam to soil.

Another embodiment of the invention comprises a method for controlling aquatic weeds by applying tabtoxinine-β-lactam to water, including ponds, lakes, streams, rivers and estuaries.

For purposes of this application, a weed is "any plant that is objectionable or interferes with the activities or welfare of man" at the location where it is growing. A herbicide is a "chemical used to control, suppress, or kill plants, or to severely interrupt their normal growth processes." Herbicide Handbook of the Weed Society of America, Fifth Edition (1983), xxi-xxiv.

Description Of The Preferred Embodiments

Tabtoxin and tabtoxinine-β-lactam can be produced by chemical synthesis according to methods known to those skilled in the art. See, e.g., J.E. Baldwin, et al., "Stereospecific Synthesis of Tabtoxin," Tetrahedron, 40(19):3695-3700 (1984); J. Baldwin et al., "Synthetic Studies on Tabtoxin. Synthesis of a Naturally Occurring Inhibitor of Glutamine Synthetase, Tabtoxinine-β-lactam, and Analogues," Tetrahedron 42(12):3097-3110 (1986); R.E. Dolle et al., "Enantiospecific Synthesis of (-)-tabtoxinine β-lactam," J. Org. Chem., 57:128-132 (1992). Tabtoxin may be isolated from culture media in which certain pathovars of Pseudomonas syringae have grown. Methods for purification of tabtoxin produced by fermentation are known in the art and have been described in the literature. E.g., W.W. Stewart, Isolation and proof of structure of wildfire toxin, Nature 229:174-178 (1971). A method for the isolation of tabtoxinine-β-lactam produced by Pseudomonas syringae ATCC

#55090 is described below in the Examples. The purified tabtoxinine-β-lactam may be stored as a dry powder.

A novel strain of Pseudomonas syringae (isolated from a tobacco plant in Maryland by Dr. Arvydas Grybauskas of the Department of Botany, University of Maryland, College Park, Maryland) which produces tabtoxinine-β-lactam was deposited on August 24, 1990, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, and was assigned accession number ATCC #55090.

This strain was originally selected for its ability to infect various target weeds. It is particularly virulent on tobacco. Tobacco plants (Nicotiana tabacum L.) were used periodically to confirm the virulence of this strain of Pseudomonas syringae. The fatty acid profiles of this bacterium resemble the fatty acids of Pseudomonas syringae pv. papulans. However, this pathovar differs from Pseudomonas syringae pv. papulans in its virulence toward tobacco and in its production of the toxin described here, which differs from any toxin reported to be produced by Pseudomonas syringae pv. papulans. A. Evidente et al., Isolation of β-Phenyllactic Acid Related Compounds from Pseudomonas syringae, Biochemistry 29(5):1491-1497 (1990).

A wide variety of liquid media can be used to cultivate Pseudomonas syringae ATCC #55090. Compositions of media appropriate for growing microorganisms are well-known in the art, as are methods for growing microorganisms and liquid media on a commercial scale. See, e.g., E.L. Demain and N.A. Solomon (Eds.), Manual of Industrial Microbiology and Biotechnology, Washington, D.C., American Society for Microbiology, 1986. Preferably, the media for cultivating Pseudomonas syringae ATCC #55090 and other TBL-producing strains should contain soyflour. Optimization of other parameters such as temperature, time of incubation, degree of aeration and agitation, etc., can also be determined by a person of ordinary skill by means of simple comparative testing.

Well-known techniques for the isolation or selection of mutants, either naturally occurring or induced, can be used to obtain mutants and variants of Pseudomonas syringae ATCC #55090 that produce greater quantities of tabtoxinine-β-lactam. This can also be done by modifying the microorganism using well-known techniques of classical strain improvement, genetic engineering, recombinant DNA and fermentation research.

Classical strain improvement involves the use of mutagens such as nitrosoguanidine (NTG), hydroxylamine, ethyl methanesulfonate (EMS), diethylsulfate (DES), and ultraviolet light (UV light). The bacterial cells to be mutagenized are exposed to the mutagen for the desired period of time and grown overnight on Petri plates containing KB media. Individual colonies are then selected from the plates and tested for increased tabtoxinine-β-lactam production. Once the new strain has demonstrated increased tabtoxinine-β-lactam production, it becomes the new parent strain and is exposed to the mutagen. This cycle is repeated until the desired level of production is achieved.

Strains for tabtoxinine-β-lactam production can also be improved by genetic engineering, as is known to those skilled in the art. Basically, genes affecting tabtoxinine-β-lactam production can be isolated, allowing for manipulation of expression to optimize toxin production. More specifically, genes affecting tabtoxinine-β-lactam production may be isolated from the current production strain, other tabtoxin or tabtoxinine-β-lactam producers, other related Pseudomonas species, or unrelated organisms. Such genes include, for example, genes for tabtoxinine-β-lactam biosynthetic enzymes, genes for regulatory factors affecting biosynthetic gene expression, genes for resistance to tabtoxinine-β-lactam, genes affecting the stability of tabtoxinine-β-lactam, or genes for enzymes involved in primary metabolism involved in availability of toxin precursors. These genes may be isolated by means known to those skilled in the art, such as transposon mutagenesis and tagging, by genetic

selections, by DNA or protein homology with known sequences, or other such means. Expression of tabtoxinine-β-lactam production-related genes may be manipulated, for example, by controlling the level of transcriptional regulatory factors, by changing gene copy number using either plasmids or chromosomally-integrated copies, by using other promoter sequences, by deleting genes, or by similar means.

Then, the genes of interest can be transferred to a suitable production host, such as the current production strain, a different toxin producer, a related Pseudomonas species or an unrelated bacterium or fungus. The engineered genes may be moved into the production host by those methods known in the art, such as transformation or electrotransformation using phage or plasmid vectors, or by conjugation or transfection.

Preferably, the production strain may be improved by isolating the genes for biosynthesis of tabtoxinine-β-lactam, replacing the promoter(s) with strong inducible heterogeneous promoters, such as lac or tac, and then moving the new biosynthetic operon into Escherichia coli on a high copy number plasmid, thus allowing for high levels of production in a fermenter upon induction.

To isolate tabtoxinine-β-lactam secreted into the culture medium, the bacteria should be removed from the medium. The microorganisms can be removed by well-known methods such as sedimentation and microfiltration or ultrafiltration or reverse osmosis. If desired, the organisms may be killed by well-known methods such as heating, irradiation, or the use of bacteriocidal chemicals. The dead bacteria can be removed from the conditioned medium by sedimentation, filtration or other means.

The cell free fermentation medium can then be passed through a column containing a cation exchange resin which binds the toxin and allows most of the spent media components to pass through and be discarded. Examples of cation exchange resins that may be used are Dowex 50W or Amberlite 200 in the H^+ form. Tabtoxinine-β-lactam can be eluted from

the cation exchange resin with an appropriate buffer, concentrated by various drying methods such as lyophilization, rotary evaporation or spray drying, and then formulated for application.

The present invention is also directed to salts and derivatives of tabtoxinine-β-lactam. Derivatives of the amino group may be prepared by protonation, alkylation, and/or acylation. Protonated derivatives are prepared by treatment with acids such as sulfuric acid, phosphoric acid and carboxylic acids such as acetic acid, propionic acid and benzoic acid. Alkylated derivatives are prepared by well-known alkylation processes such as treatment of the amine with methyl iodide, bromide, or chloride. Other alkyl and aryl halides can also be employed, such as isopropyl chloride, n-butyl chloride and cyclohexyl chloride. Acylation is accomplished by well-known acylating methods, which include acylation with sulfuric acid derivatives, phosphoric acid derivatives and carboxylic acid derivatives such as acetic anhydride, acetyl chloride, benzoyl chloride and other carboxylic acid chloride or halide derivatives. Of particular interest are derivatives of the amino group which provide increased stability to the toxin and/or increased lipophilicity to the molecule to aid in leaf and membrane permeability.

The herbicidal composition of the invention comprises not only the free acid of tabtoxinine-β-lactam, but also its salts. Preferably, salts of tabtoxinine-β-lactam are used as herbicides. The cation should preferably be one that is biodegradable and does not pose environmental hazards. Such salts are known to the art and include salts of alkali metals, alkaline earth metals, or sulfur, phosphorus or nitrogen based salts such as sulfonium, phosphonium, and ammonium as well as organic ammonium salts such as alkylammonium and arylammonium derivatives.

The term "alkali metal" encompasses lithium, sodium, potassium, cesium and rubidium; and the term "alkaline earth

metal" includes beryllium, magnesium, calcium, strontium and barium.

Organic ammonium salts are those prepared from low molecular weight organic amines, i.e. having a molecular weight below about 300, and such organic amines include the alkyl amines, alkylene amines and alkanol amines, such as methylamine, ethylamine, n-propylamine, isopropylamine, n-butylamine, isobutylamine, sec-butylamine, n-amylamine, iso-amylamine, hexylamine, heptylamine, octylamine, nonylamine, decylamine, undecylamine, dodecylamine, tridecylamine, tetradecylamine, pentadecylamine, hexadecylamine, heptadecylamine, octadecylamine, methylethylamine, methylisopropylamine, methylhexylamine, methylnonylamine, methylpentadecylamine, methyloctadecylamine, ethylbutylamine, ethylheptylamine, ethyloctylamine, hexylheptylamine, hexyloctylamine, dimethylamine, diethylamine, di-n-propylamine, diisopropylamine, di-n-amylamine, diisoamylamine, dihexylamine, di-heptylamine, dioctylamine, trimethylamine, triethylamine, tri-n-propylamine, triisopropylamine, tri-n-butylamine, triisobutylamine, tri-sec-butylamine, tri-n-amylamine, ethanolamine, n-propanolamine, isopropanolamine, diethanolamine, N,N-diethylethanolamine, N-ethylpropanolamine, N-butylethanolamine, allylamine, n-butenyl-2-amine, n-pentenyl-2-amine, 2,3-dimethylbutenyl-2-amine, di-butenyl-2-amine, n-hexenyl-2-amine and propylenediamine, primary aryl amines such as aniline, methoxyaniline, ethoxyaniline, o,m,p-toluidine, phenylenediamine, 2,4,6-tribromoaniline, benzidine, naphthylamine, o,m,p-chloroaniline, and the like; heterocyclic amines such as pyridine, morpholine, piperidine, pyrrolidine, indoline, azepine and the like.

Of particular interest are those derivatives of tabtoxinine-β-lactam, such as those listed above, that will impart lipophilicity to aid in membrane permeability.

To use tabtoxinine-β-lactam as a herbicide, it may be applied topically to the foliage of weeds or to soil, by methods of application well-known in the art, such as

spraying. The toxin may be isolated and used in its purified form, or it may be applied in unpurified form, for example, as a constituent of culture medium in which Pseudomonas syringae ATCC #55090 and other TBL-producing strains have grown. Preferably, for most types of weeds the amount of toxin applied in is the range of from 0.3 kg/ha to 12 kg/ha, more preferably 1.3 kg/ha to 2.0 kg/ha.

The toxin may be applied to the foliage of weeds or soil as a buffered solution. Buffers may be any of the buffers known to those skilled in the art. Preferably, the buffer is a 0.1 M salt solution. More preferably, the buffer is a 0.1 M solution of potassium phosphate (K_2PO_4).

Application of tabtoxinine-β-lactam may be post-emergent, as a nonselective contact herbicide. Application may also be pre-emergent, by applying the toxin directly to soil in which weed growth is to be controlled. The invention can be used to control weeds in an agricultural setting and can also be used for nonagricultural applications. For example, the invention can be used to control weeds in residential applications and as a contact herbicide for the management of roadside vegetation.

In general, it was found that broad leaf weeds were somewhat more sensitive to tabtoxinine-β-lactam than narrow leaf weeds or grass weeds. Some of the more sensitive weeds included hemp sesbania, lambsquarters, field bindweed, pigweed, sicklepod, clover and carpet weed.

Tabtoxinine-β-lactam begins to act soon after application. In some field experiments, signs of injury to the weeds such as water soaking of the leaves was observed within a few hours after the toxin was applied. In most experiments the degree of injury was rated quantitatively at 13-16 days after application of tabtoxinine-β-lactam, since long lasting control of weeds is of commercial interest.

The herbicidal effects of tabtoxinine-β-lactam involve processes that require light. When tabtoxinine-β-lactam was sprayed on plants and the plants are kept in the dark overnight or for one or two days, no injury was observed.

Signs of injury begin to appear within a few hours after the plants were returned to the light without application of additional tabtoxinine-β-lactam.

The invention can also be used as a harvest aid to defoliate crops such as potatoes, peanuts and cotton. Several major crops often require defoliation prior to harvesting. This reduces the amount of dirt, leaves and other contaminants in the final product. For example, as much as 75% of the cotton harvested in the U.S. is treated with defoliants. The current defoliants are chemically based and pose potential health and environmental risks. In addition, undesirable odors are often noticeable for several days after some of the commonly used defoliants are applied.

Desiccants are used as a harvest aid to eliminate unwanted green plant material and dry out or harden off seed crops such as alfalfa and clover prior to harvesting. The herbicidal composition of the invention can be used as a defoliant or desiccant on some of the major crops such as potatoes, peanuts and cotton, thus eliminating some of the undesirable side effects associated with the current chemical based products.

Compositions for practice of the invention can be formulated in numerous ways, including flowables, dry flowables, water dispersible granules, emulsified or liquid concentrates, or encapsulated using various techniques and materials.

The toxin can also be used in combination with other chemical herbicides to control weeds. Many chemical herbicides such as sulfosate (TOUCHDOWN®), glyphosate (ROUNDUP®), alachlor (LASSO® or MICRO-TECH®), 2,4-D, and dicamba (BANVEL®) have shortcomings in either the speed of activity or the weed control spectrum. By combining the toxin with chemical herbicides in either tank mixes or package mixes, improved performance can be achieved. For example, a tank mix combination of either sulfosate or glyphosate with the toxin improves the herbicidal efficacy of both the chemical and the toxin, thus reducing the amounts

that must be applied. Furthermore, the speed at which weed control is achieved is increased by 50 to 100% when compared to the herbicide alone.

Herbicides that can be combined with tabtoxinine-β-lactam in improved herbicidal compositions are listed in Table 1. Examples of additional chemical herbicides that can be used are described in Herbicide Handbook of the Weed Society of America, Sixth Ed. (1989), Farm Chemicals Handbook, Willoughby, Ohio: Meister Publishing Co., (1990), and in H. J. Lorenzini and L.S. Jeffrey, Weeds of the United States and Their Control, N.Y.: Van Nostrand 1987, Table 1.9.

Herbicides comprising tabtoxinine-β-lactam may also contain adjuvants such as surfactants and suspension agents. Examples of adjuvants that can be used include spreaders such as ORTHO-77® (Chevron Chemical Co.), ATTAGEL-40® and X-77®, and organosilicones such as SILWET L-77®, SILWET L-7607®, X2-5309 (SYLGARD 309®) (Dow Corning), Genapol LRO (Hoechst-Roussel Agri Vet Company), X2-5395 (Dow Corning), Kinetic, and Dyne-amic (Helena). According to the manufacturer, active agents in X-77® are alkylarylpolyoxyethylene glycols, free fatty acids and isopropanol. Polyoxyethylene surfactants are preferably employed as adjuvants, more preferably alcohol polyoxyethylene surfactants such as MARLIPAL-34®. Crop oils that can be used as adjuvants include Sure Crop Agridex, Crop Surf, CIDE-KICK®, and Crop Oil Concentrate (Crop Surf Spray Oil, Universal Cooperatives, Inc., 7801 Metro Parkway, Minneapolis, MN 55440). Other adjuvants include SOY-DEX®, AGRI-DEX®, TRITON AG-98®, STEROX®, ammonium sulfate, Atlas G-3780A, urea, vegetable oil, Tween A and invert emulsions. Some adjuvants and the chemical compositions of their major components are listed in Table 2.

Certain adjuvants have proven to be particularly advantageous for use with tabtoxinine-β-lactam. When isolated toxin is applied to weeds with these preferred adjuvants, the degree of weed control is unexpectedly greater than is seen with other adjuvants. The preferred adjuvants

are neutral surfactants and neutral organosilicone surfactants. Examples of currently preferred adjuvants are ORTHO-77®, SYLGARD-309®, SILWET-7604® and SILWET-7614®.

Phloem or xylem translocation is a desirable property for a herbicide. See J.R. Hay, "Herbicide Transport in Plants," L.J. Audus (ed.), Herbicides, Physiology, Biochemistry, Ecology, 2nd Ed., Vol. I., N.Y.: Academic Press, 1976, pp. 365-396. Total weed control can be achieved much more readily with a phloem- or xylem-mobile herbicide as compared to non-mobile contact herbicides. As illustrated below, we have demonstrated that when tabtoxinine-β-lactam is painted on a single leaf of a plant it moves throughout the plant by vascular transport and produces a high degree of injury to the entire plant.

Another potential use for this invention is as an antibiotic. Monobactams such as tabtoxinine-β-lactam that are known to inhibit glutamine synthetase may have utility as antibiotics. Preliminary tests have indicated biocidal activity of both Gram-positive and Gram-negative bacteria. Because of the high activity of the antibiotic against selected strains, the compound may find utility in controlling pathogens of medical, veterinary and agricultural importance. In addition, based on the mode of action the compound may find utility for controlling fungi and arthropods.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be apparent from the description or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the methods and combinations particularly pointed out in the appended claims. The following general description is offered by way of explanation and illustration, and not by way of limitation.

The following examples are offered by way of illustration of the preferred embodiments with the true scope of the invention being indicated by the claims which follow.

EXAMPLE 1. Small Batch Culture of Pseudomonas Syringae ATCC #55090

Seed stocks of stationary phase Pseudomonas syringae ATCC #55090 were maintained in liquid culture in a modified King's B medium (Table 3). A two liter flask containing 500 ml of King's B medium was inoculated with 0.5 ml of Pseudomonas syringae ATCC #55090 and were grown overnight at 28°C on an orbital shaker at 180 rpm.

For smaller batches, fermentation was carried out in a 14 liter New Brunswick Microferm fermentor (10 liter working volume). The 500 ml seed medium was inoculated into 10 liters of fermentation medium (UWF) (Table 4). Fermentation was carried out at 25°C for 48 h to 72 h with an agitation rate of 400 rpm and an air flow rate of 14 liter/min. pH was maintained between 6.5 and 6.8. After 24 h of fermentation, a nutrient spike was added to the vessel consisting of 50 g of fructose and 25 g of KNO_3.

EXAMPLE 2. Large Batch Culture of Pseudomonas Syringae ATCC# 55090

To obtain greater quantities of toxin, the fermentation was also carried out in batches of 350 liters. Seed cultures (S_1) were prepared by inoculating 0.5 ml of a stationary phase culture of Pseudomonas syringae ATCC #55090 into two liter flasks containing 500 ml of King's B Medium. The flasks were shaken overnight at 180 rpm and 28°C. Two S_1 seed cultures were used to inoculate two Microferm fermentors each containing 10 liters of King's B Medium (S_2). The S_2 seed cultures were grown for 7 h at 25°C, 400 rpm, with an air sparge of 1 vvm (volume of air spray/volume of fermentor vessel/min). The pH was not controlled. Twenty liters of S_2 was transferred to a B. Braun 500 liter free-standing fermentor with a 350 liter working volume. The fermentation was carried out in 350 liters of UWF medium for 67-72 h at

25°C with an agitation rate of 150 rpm and an air sparge of 1 vvm, with pH maintained between 6.0 and 6.8. A one time nutrient spike was added after 24 h consisting of 5 kg of glucose.

In a preferred variation of the method described, medium F1-12 or medium F1-16 (Table 4a) is employed as the fermentation medium.

EXAMPLE 3. Isolation of a Herbicidal Composition From Culture Media

After incubation, medium was centrifuged to remove the cells using a Sorval RC5C centrifuge at 8000 r.p.m. for 8 minutes at 10°C with a GSA rotor. Any cells that remained suspended in the supernatant were removed by microfiltration through a 0.2μm filter. For larger volumes of medium (10 liters or more), cells were removed by centrifugation in a Sharples (Model AS16P8) continuous feed centrifuge fitted with a tubular bowl with a flow rate of 2-3 liters/min and a centrifuge speed of 15,000 rpm (approximately 20,000 x g).

The cell free fermentation beer was adjusted with HCl to a pH of 4.0 and passed through a cation exchange resin. Any strong cation exchange resin such as Dowex 50W or Amberlite 200 in the H^+ form can be used. Amberlite 200 in the H^+ form is preferred. The column size was adjusted according to the volume of fermentation beer to maintain the ratio of fermentation beer to resin bed volume at 10:1.

After passage through the column the spent medium was discarded. The buffers listed below were used to elute the phytotoxin from the resin bed. A ratio of 5 volumes of buffer to 1 bed volume as used. Buffers 2 and 3 are preferred. The eluate was adjusted to pH 4.0 with HCl and stored at 5° C. Concentration steps included rotary evaporation at 40° C under vacuum followed by lyophilization. The dry product was desalted with three rinses of dry methanol, filtered, and the residue discarded. The remaining filtrate was reduced by rotary evaporation and the final extract was stored at -20° C and pH 4.0. When stored dry,

the powder is redissolved in water or methanol immediately before use as a herbicide.

Buffer	Water: methanol Concentration	pH
1) 0.4M KCl	50:40	7.0
2) 0.4M NH_4HCO_3	30:70	8.5
3) 0.4M K_2HPO_4	50:50	7.5
4) 0.4M $K_2C_2H_3O_2$	20:80	7.0
5) 0.4M $KHCO_3$	40:50	9.1
6) 0.4M NaCl	30:70	7.0

TBL can be assayed with a Beckman model 126AA amino acid analyzer or similar instrument. Using a sodium ion exchange column and sodium citrate buffers, TBL has a retention time of approximately ten minutes using a standard method for amino acids. Glycine may be used as a standard for quantitative analysis of TBL.

In another method of purification, the culture medium was adjusted to pH 4.0 with HCl, and passed through a column containing activated carbon. The herbicidally active material was eluted from the activated carbon with methanol acidified to a pH of 4.2. The resulting eluate was reduced in volume, lyophilized, dissolved in a minimal amount of water and stored at -20° C at pH 4.0.

An alternative method of isolation involves direct lyophilization of the fermentation beer. After lyophilization, the dry product is extracted three times with dry methanol and the extract volume reduced under vacuum to a workable volume. The concentration of tabtoxinine-β-lactam is determined in the final product.

EXAMPLE 4. Identification of the Toxin

The toxin was identified by ^1H nuclear magnetic resonance (NMR) spectroscopy of a purified sample. The purified sample was prepared for analysis by thin layer chromatography (TLC) on Whatman silica gel 60A (250μm) TLC

plates. Small quantities (approximately 1μg) of crude toxin were spotted on the TLC plates in discrete spots. Approximately 25 plates with 30 spots per plate were required to obtain sufficient toxin for analysis. The TLC plates were developed in butanol:acetic acid:water (80:20:20). After drying, the silica on the area of the plate containing the toxin was removed and extracted with methanol at pH 4.5. The methanol was removed under vacuum and the dry product suspended in 1 ml of D_2O and allowed to sit for 30 min at 22°C. The sample was then frozen, lyophilized, resuspended in D_2O, and analyzed by ^1H NMR spectroscopy (500MHz, Bruker AMX-500). An aliquot of the purified toxin was tested in a bioassay to ensure that it retained its biological activity.

Table 5 is a comparison of the ^1H NMR spectrum of the purified toxin and a chemically synthesized sample of tabtoxinine-β-lactam (Dolle et al., J. Org. Chem., 57:128-132 (1992)) confirming that the toxin is TBL.

EXAMPLE 5. Demonstration of Herbicidal Activity in the Greenhouse

The effects of the purified toxin were evaluated in the greenhouse on weed species selected as being weeds of economic importance in row crops, residential and industrial markets and as representing a spectrum of plant families. The effects were also tested on tomato, another representative broadleaf plant.

Methods for testing the effects of the toxin on weeds are described in WO/9208357. In other tests the toxin was applied using a hand sprayer technique. The isolated toxin was dissolved in water immediately prior to use. The pH of the solution was kept below 7.0. The preferable range is 4.0-5.0. Extracts were suspended in 8 ml of water and applied to 1.4 square feet (0.13 m^2) using a DeVilbiss sprayer pressurized with compressed CO_2 to atomize the liquid. The spray was delivered approximately 8 to 10 inches (10-20 cm) above the plant foliage at a rate of 66 gallons per acre (741 L/ha). Two replications were used and arranged

in a randomized complete block design. Narrowleaf weeds (grasses) were approximately 5 to 10 inches tall (13-25 cm) while broadleaf weeds were approximately 4 to 8 inches (10-20 cm) tall.

For both greenhouse and field tests, application rates of the extract varied depending on the efficacy of the extraction process and how significantly the extract was concentrated during the final drying step. Rates were set based on the initial amount of fermentation beer that was used to prepare the extract. Rates varied from 0.5 to 1.0 liter of fermentation beer/square foot (.107 m^2) depending on the target weed species. Improvements in the extraction process and development of an analytical technique have provided a more precise estimate of the application rate of 0.3 to 2.0 kg/ha. Examples of application rates that resulted in commercial levels of weed control are:

Weed	Application rate equivalent to liters fermentation beer/m^2	Phytotoxin dose kg/ha
Lambsquarters	1.0	0.6
Pigweed	1.0	0.6
Carpet weed	0.5	0.3
Morningglory	5.0	1.7
Field bindweed	5.0	1.7
Barnyardgrass	5.0	1.7
Giant foxtail	5.0	1.7
Johnsongrass	5.0	1.7
Green foxtail	5.0	1.7

Injury was rated on a scale of 0-100 with 0 = no injury and 100 = plant death, as described in Research Methods in Weed Science, 3rd Edition, Southern Weed Science Society, 1986, p. 37. This type of rating evaluation is widely used to evaluate the effects of herbicides on weeds.

Various adjuvants were applied with the herbicidal extract. ORTHO X-77® was applied at 0.25 to 0.5 % (v/v). The vehicle alone (water or water plus surfactant) served as a control. The experimental design was a randomized complete

block design with at least 2 replications per treatment and 2 to 8 species per replication.

The results of applying different amounts of toxin are shown in Table 6. Injury was evaluated 13 days after treatment. Water containing only adjuvant produced no plant injury. The extract from the fermentation beer produced substantial plant injury to all plants tested. The degree of injury increased with increasing amounts of toxin extract.

In control experiments, culture medium in which no bacteria had grown was subjected to the same purification procedures. Extracts of control medium formulated with 0.5% X-77 were tested on the same panel of weeds. The extract of the control medium caused little or no injury to the weeds. Thus, the herbicidal activity in the conditioned medium is produced by the bacteria and is not a component of the original medium.

EXAMPLE 6. Herbicidal Action on Weeds in the Field

The effects of the extract were tested on weeds in the field. Field experiments were conducted at the Crop Genetics International research farm at Henderson, Maryland. Spray solutions were prepared by adding the phytotoxin extract and adjuvant to an appropriate amount of water. Field treatments were applied using a CO_2 pressurized backpack sprayer equipped with 004 flat fan nozzles delivering 233.7 liters/ha at a speed of 93.9 m/min. Field studies typically included three or four broadleaf weed species having 4 to 6 true leaves and three or four narrowleaf species 5 to 10 cm in height. Plots were 0.3 x 2 m with 25 cm row spacing.

The results are shown in Table 7. At 14 days after treatment, a dose of 0.63 kg/ha of phytotoxin produced injury in all of the species. Application of 0.84 kg/ha of toxin produced levels of plant injury approaching 100% for most of the species tested.

EXAMPLE 7. Vascular Transport of Toxin

Experiments were conducted to determine whether the phytotoxin acted locally or spread from the site of application throughout the plant.

Either one new leaf or one mature leaf of each plant was painted with a mixture of phytotoxin and 0.5% (v/v) surfactant. Water and surfactants served as a control treatment. The plants were returned to the greenhouse and injury to the entire plant (not only the treated leaf) was evaluated 11 days after treatment. The results in Table 8 show that after the toxin was applied to one leaf it moved throughout the plant and produced a high degree of injury of the entire plant.

EXAMPLE 8. Effects of Adjuvants

Examples of some adjuvants that may be combined with the toxin to enhance herbicidal action are listed in Table 2. The effects of using different surfactants in combination with the toxin are shown in Table 9. Injury was evaluated 16 days after treatment. For most weeds, the injury score was double or more when the toxin was applied with X-77® (0.5-1.0% v/v) or SYLGARD-309® (0.1-0.25% v/v). Addition of 1-3% glycerin as a humectant did not enhance the herbicidal action of the phytotoxin alone or of the phytotoxin with SYLGARD-309®.

A second experiment comparing the effects of phytotoxin applied alone and phytotoxin applied with various adjuvants or other substances is shown in Table 10. In Table 10, results were evaluated 14 days after treatment. Comparison of the data in Tables 9 and 10 for the phytotoxin alone and for the phytotoxin plus ORTHO X-77® illustrates the degree of reproducibility in these experiments. Neutral organosilicone surfactants (SILWET-7604 and SILWET-7614) were particularly effective in enhancing the activity of the toxin.

EXAMPLE 9. Combination of Toxin with Chemical Herbicides

The toxin can be combined with chemical herbicides to enhance weed control. Effects of combining the toxin with sulfosate are shown in Table 11.

EXAMPLE 10. Effects of Phytotoxin on Different Types of Weeds

Table 12 provides examples of the spectrum of weeds that can be controlled with the phytotoxin.

EXAMPLE 11. Effects on Duckweed

Duckweed (Lemna minor) is a small aquatic weed that can be used as an indicator of herbicide activity on aquatic weeds or as a bioassay for herbicides and plant growth regulators. Duckweed is grown in water containing minimal salts such as Hoagland's growth solution at pH 4.0. The plants are maintained in a stock culture in a growth chamber under continuous light (approximately 250 $\mu E\ m^{-2}\ sec^{-1}$) and 20°C.

The bioassay is conducted in 24 well culture plates as follows: Under sterile conditions, 1.0 ml of medium was added to each well along with two duckweed frond doublets of equivalent size from the stock culture. Treatments were added to each well as indicated in Table 13. The plates were returned to the growth chamber for 5 days.

After 5 days, the plates were removed from the growth chamber, the medium was removed by aspiration, and 1.0 ml of methanol was added. The plates were allowed to stand for 4 hours at room temperature and the concentration of chlorophyll in each well was determined by measuring the absorbance of a 200 μl sample at a wave length of 650 nm in a spectrophotometer. The reduction in chlorophyll concentration as compared to the control plants is indicative of the amount of injury caused by the toxin or herbicide. The results are presented in Table 13.

In a separate experiment, the activity of TBL was compared to other herbicides using duckweed. The level of activity of TBL in the duckweed bioassay was unexpected when

compared to other well known herbicides and phytotoxins (Table 17). These data demonstrate the potential utility of TBL as a weed control agent.

EXAMPLE 12. Derivatives of Tabtoxinine-β-Lactam

A. Esters

Ester derivatives of tabtoxin are prepared in the following manner, illustrated with the methyl ester of tabtoxinine-β-lactam: 5 g of tabtoxinine-β-lactam is dissolved in 25 ml of anhydrous methanol and 0.2 g of concentrated sulfuric acid is added in one portion. The homogenous solution is allowed to stir at room temperature for 12 hours. Dilute sodium hydroxide solution is added to bring the pH of the mixture to 6.5. The solution is evaporated to dryness and the methyl ester is isolated by standard techniques.

An alternative method of preparing ester derivatives of tabtoxin follows: In a three-neck round bottom flask equipped with a reflux condenser and a gas inlet tube are placed 20 g of tabtoxinine-β-lactam and 300 ml of absolute methanol. A stream of anhydrous HCl gas is passed through the solution for one minute while maintaining the temperature below room temperature. The reaction mixture is concentrated *in vacuo*. 50 ml of absolute methanol are added to the resulting product and the solution concentrated *in vacuo* to dryness.

B. Amides

This example illustrates the use of ethyl chloroformate as the acid chloride to derivatize the amine group at C-2'. Tabtoxinine-β-lactam is dissolved in water and the pH of the solution is adjusted to 7.5. The pH of the solution should be maintained at 7.5 during the course of the reaction. The pH must be maintained below 8.0. The reaction could also be run by maintaining a lower pH such as pH 6.5, but the reaction proceeds faster at pH 7.5.

Excess ethyl chloroformate is added dropwise to this solution at 0°C until no amine can be detected by ninhydrin

reaction. The pH is then adjusted to pH 5.5, the solution is evaporated to dryness and purified by standard techniques.

C. Tosyl sulfonamides

In a 25 ml round bottom flask in an ice bath are placed 8 ml water, 4 ml tetrahydrofuran and 0.65 g of tabtoxinine-β-lactam. To this mixture is added with stirring 1.45 g tosyl chloride in small portions over about 30 min. The pH is maintained between 7.0 and 7.5. After the reaction is complete as measured by thin layer chromatography, the tetrahydrofuran is removed *in vacuo* and 10 ml of water are added. The aqueous solution is washed twice with 25 ml portions of ether and the solution acidified by addition of concentrated HCl until the sulfonamide hydrochloride salt precipitates. The product is isolated by standard techniques.

D. Trifluoroacetates

In a 25 ml round bottom flask cooled in an ice bath 2.0 g of tabtoxinine-β-lactam is added to 6.0 ml of trifluoroacetic anhydride with stirring. The resulting mixture is allowed to stir until the reaction is complete as determined by comparative TLC. The reaction mixture is concentrated in vacuo, then 50 ml cold water is added and the reaction is allowed to stir until precipitation is complete. The product is isolated by standard techniques.

E. Carbobenzoates

To a suspension of 4.2 g sodium bicarbonate in 25 ml of water in a 100 ml round bottom flask are added 2.5 g of tabtoxinine-β-lactam and the resulting mixture is stirred vigorously. To this mixture is added 3.74 g of benzyloxy chloroformate in small portions over about 30 min, and the mixture is left to stir at room temperature for about 1 hour. Once the reaction is complete, the reaction mixture is washed twice with 25 ml portions of ether. The aqueous phase is then acidified to pH 4 and the desired product extracted with ethyl acetate (3 x 50 ml). The organic phases are combined, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The product is purified by standard techniques.

EXAMPLE 13. Antibiotic Activity

Antibiotic activity can be demonstrated by growing ATCC strain #55090 on petri plates with various indicator strains or by exposing the target strains to various concentrations of toxin.

In the first assay, indicator strains were suspended in a minimal media such as PMS and allowed to solidify in plates. The producer strain (Pseudomonas syringae ATCC strain #55090) was inoculated into the minimal medium containing the indicator strain. Cultures were incubated overnight at 28°C and colony diameter was measured. The resulting inhibition of bacteria is summarized in Table 14.

In other tests, plates containing bacterial indicator strains were prepared as described above. Sterilized filter paper discs (5mm) were treated with 20μL of the appropriate dilution of toxin, allowed to dry and applied to the plates. The plates were incubated overnight at 28°C and the halo diameter measured to indicate antibiotic activity. (Table 15).

EXAMPLE 14. Genetic Engineering for Strain Improvement

To improve the production strain, a broad host range multi-copy plasmid containing the toxin biosynthetic genes is obtained and moved into a toxin producing isolate, thereby increasing the copy number of the biosynthetic genes and potentially the level of production. This is accomplished by:

Step 1: Selection of a drug resistant toxin producing recipient.

In order to select for transconjugants from the bacterial mating described in Step 2, it is necessary to have a selectable marker present in the recipient. To do this, the current production strain is plated on KB (King et al., J. Lab. Clin. Med. 44:301-307 (1954)) plus rifampicin (100 μg/ml), incubated at 28°C and resistant colonies isolated

and purified as described in Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1972. Rifampicin resistant isolates are checked for maintenance of toxin production by bioassay using toxin sensitive Escherichia coli as described in Gasson, Appl. Environ. Microbiol. 39:25-29 (1980).

Step 2: Move plasmid containing the toxin biosynthetic genes into the recipient.

The entire tabtoxin biosynthetic region from Pseudomonas syringae BR2 is contained on the cosmid pRTBL823, which also contains a tetracycline resistance marker (Kinscherf et al., J. Bacteriol. 173:4124-4132 (1991)). In order to move this plasmid into the recipient strain, a triparental mating between the donor strain E. coli DH5α(pRTBL823), the helper strain E. coli pRK2013 (ATCC #37159) and the recipient strain is carried out as described in Ditta et al., Proc. Natl. Acad. Sci. USA 77:7347-7351 (1980). Transconjugants (colonies arising from the movement of pRTBL823 into recipient cells) are selected on KB plates containing tetracycline (10 μg/ml) and rifampicin (100 μg/ml) at 28°C. After restreaking transconjugants on KB + tet + rif, isolates are checked for kanamycin sensitivity (the helper plasmid pRK2013 (ATCC #37159), which should not be present in the transconjugants, contains a kanamycin resistance marker) and toxin production by bioassay.

The resulting isolates have an increased copy number of the toxin biosynthetic genes, including the native genes present in the current toxin producer and multiple copies of the BR2 genes present on multiple copies of the plasmid. When grown under tetracycline selection to maintain the plasmid, such isolates produce increased amounts of toxin.

Many variations of this procedure are possible and would be obvious to persons skilled in the art. These include the use of other recipients, the subcloning of the BR2 biosynthetic genes onto higher copy number plasmids, the modification of the biosynthetic genes by deletion, mutation,

substitution or rearrangement prior to movement into the recipient, and the use of genes involved in the regulation of the biosynthetic genes or pathway.

In a variation of the method described, the recipient Pseudomonas strain is prototrophic, and the donor and helper E. coli strains are both auxotrophic, so that growth on minimal media is used as the selectable phenotype of the recipient (instead of rifampicin resistance). For this, a triparental mating between the donor strain E. coli HB101 (pRTBL823), the helper strain E. coli (pRK2013 [ATCC #37159], and the recipient strain Pseudomonas syringae pathovar tabaci 11527 [ATCC #11527] is carried out. Cultures of the three strains are grown in liquid media to mid-log phase, the cells harvested by centrifugation, and the cells re-suspended at about 10-fold higher density in M9 liquid media (Miller). Approximately equal numbers of cells of all three strains are mixed together in a total volume of 50 to 300 microliters of M9, then collected by filtration on a 0.2 micron sterile filter. The filters are placed on LB plates (Miller) at 28°C overnight. Then, the cells are collected by washing the filters with 300 to 700 microliters of PBS. Serial dilutions of this cell suspension are plated on M9 plates containing 6 to 8 micrograms/milliliter tetracycline. Transconjugants are restreaked on KB containing tetracycline (10 μg/ml) to check for retention of the fluorescent phenotype and then for toxin production by plate bioassay on an E. coli lawn. Individual colonies are miniprepped and the existence of pRTBL823 in the tranconjugants is verified.

EXAMPLE 15. Pre-emergence Activity in Greenhouse Trials

Pre-emergence herbicides are very useful to farmers by eliminating weed competition during the early phases of crop development and represent a major portion of the herbicides market. TBL was tested as a pre-emergent herbicide in the greenhouse. The concentrated TBL prepared as described in Example 3 was suspended in 8 ml of water and applied to pots at the rate of 61 mL/m^2 using a DeVilbiss hand sprayer

pressured with compressed CO_2. The pots (4" or 10 cm square) contained soil (Metro mix) and the seeds of the appropriate weed species. Two replications were used and arranged in a randomized complete block design. Injury was rated as outlined in Example 5. Results from the pre-emergence trials are shown in Table 16. Ratings were taken 19 days after treatment in experiment 1 and 16 days after treatment in experiment 2.

Pre-emergence activity as demonstrated by the results in Table 16 was unexpected and not previously reported for TBL when applied to soil.

Table 1 Examples of Herbicides that Can Be Used in Combination with Phytotoxin

Alachlor
Atrazine
Bentazon
Bialaphos
Butachlor
Butylate
Chlorimuron ethyl
Chlorsulfuron
Cinmethylin
Cyanazine
Cycloate
Dicamba
2,4-D (2,4-dichlorophenoxyacetic acid)
EPTC
Ethephon
Fenoxaprop
Fluazifop-butyl
Fomesafen
Glufosinate
Glyphosate
Haloxyfop
Hoelon
Imazapyr
Imazaquin
Imazethapyr
Linuron
Mefluidide
Metolachlor
Metribuzin
Metsulfuron
Molinate
Norflurazon
Oryzalin
Oxyfluorfen
Paraquat
Pendimethalin
Picloram
Propachlor
Propanil
Pyridate
Sethoxydim
Simazine
S,S,S-tributyl phosphorothioate
Sulfometuron
Sulfosate
Trifluralin

Table 2 Examples of Adjuvants that Can Be Combined with the Phytotoxin

Trade Name	Chemical Type or Name
SILWET -7604 -7614 -L-77	polyalkylene oxide-modified polydimethylsiloxanes
SYLGARD 309	2-(3-hydroxypropyl)-heptamethyl -trisiloxane
TWEEN 20	sorbitan monolaurate (polysarbate 20)
TWEEN 80	sorbitan monooleate (polysorbate 80)
TRITON X-100	polyoxyethylene ethers
CIDEKICK	d'limineno oil
AGRIDEX	paraffin based petroleum oil
Genapol LRO	
MARLIPAL-34	alcohol polyoxyethylene
HM 9121-A	blend of ionic and nonionic surfactants
Kinetic	blend of polyalkyleneoxide, modified polydimethylsiloxane and nonionic surfactant
Dyne-amic	blend of polyalkyleneoxide, modified polydimethylsiloxane, nonionic surfactant, and selected emulsifiers
Solulan 16	ethoxylated fatty alcohols
Solulan 25	ethoxylated fatty alcohols
Solulan C24	ethoxylated fatty alcohols
Witco P-1059	alkylaryl sulfonate
Ameroxol OE20	ethoxylated oleyl alcohol

Table 3

Modified King's B Medium

	(per liter)
Proteose peptone No. 3 (Difco*)	20.0 g
Glycerol	15.0 ml
$K_2HPO_4.3H_2O$	1.5 g
$MgSO_4.7H_2O$	1.5 g

pH 7.2

King, E.O., Ward, M.K., and Raney, D.E. Two Simple Media for the Demonstration of Pyocyanin and Fluorescin. J. Lab. Clin. Med. 44: 301-307 (1954).

*Difco Laboratories, Inc., Detroit, Michigan 48232.

Table 4 UWF Fermentation Medium

	(Per liter tap water)
Fructose	10.0 g
K_2HPO_4	1.0 g
NaH_2PO_4	1.0 g
$MgSO4.7H_2O$	0.2 g
$CaCl_2.2H_2O$	0.1 g
$FeSO_4.7H_2O$	0.02 g
KNO_3	5.0 g
MES buffer	19.5 g

pH 6.5

Table 4a

F1-12 Medium	(grams/liter)
Glycerol	15
Glucose	15
soyflour	25
NaCl	3
Ca_2CO_3	1

F1-16 Medium	(grams/liter)
Fructose	60
soyflour	25
NaCl	3
Ca_2CO_3	1

Table 5 Comparison of Chemically Synthesized TBL NMR Spectrum and the Toxin Isolated from Pseudomonas Syringae ATCC #55090

TBL (300 MHz/D_2O)*	Pseudomonas toxin (500 MHz/D_2O)
3.75 (m, 1H)	3.63 (m, 1H)
3.50 (d, 1H, J=7.0 Hz)	3.31 (d, 1H, J=6.4 Hz)
3.35 (d, 1H, J-7.0 Hz)	3.18 (d, 1H, J=6.4 Hz)
2.20-1.70 (m, 4H)	1.94-1.70 (m, 4H)

* Dolle et al., J. Org. Chem., 57:128-132 (1992).

Table 6 Effect of Toxin on Weeds Grown in a Greenhouse

		% Injury						
Treatment	Dose (ml/ft^2)	Tomato	Velvet-leaf	Jimson-weed	Crab-grass	Barnyard-grass	Giant Foxtail	Johnson grass
Control	0	0	0	0	0	0	0	0
phytotoxin	0.25	59	33	62	42	28	42	26
phytotoxin	0.5	98	42	93	73	42	74	63
phytotoxin	1.0	100	81	92	79	63	79	63

Injury was evaluated 13 days after treatment.

($1.0\ ml/ft^2 = 9.4\ ml/m^2$)

Table 7 The Effect of Phytotoxin on Weeds in the Field

		% Injury					
Treatment	Dose kg/ha	Velvet-leaf	Bird-weed	Morning-glory	Barnyard-grass	Giant Foxtail	Johnson grass
Control		0	0	0	0	0	0
phytotoxin	0.5	42	60	10	20	89	38
phytotoxin	0.75	62	46	33	95	95	60
phytotoxin	1.0	63	75	53	100	100	95
phytotoxin	1.5	99	75	90	100	99	94
phytotoxin	2.0	99	95	81	100	100	96
phytotoxin	3.0	100	89	85	100	100	98

Injury was evaluated 14 days after treatment.

Table 8 Vascular Transport of Phytotoxin Applied to One Leaf

		% Injury			
	Treatment	Velvetleaf	Jimsonweed	Tomato	Pigweed
New Leaf	Water + Surfactant	0	0	0	0
	0.7 mg/leaf	80	50	50	95
	1.4 mg/leaf	98	98	98	98
Mature Leaf	Water + Surfactant	0	0	0	0
	0.7 mg/leaf	98	98	98	98
	1.4 mg/leaf	98	98	98	98

Injury was rated 11 days after application of the toxin to one new or one mature leaf. Injury ratings (0 = no injury, 100 = death) are for entire weed and not just the treated leaf.

Table 9 Effects of Combinations of Toxin and Adjuvants

	% Injury					
Treatment	Velvetleaf	Jimsonweed	Ragweed	Tomato	Barnyardgrass	Giant foxtail
Water	0	0	0	0	0	0
Phytotoxin	37	100	62	10	27	35
Phytotoxin X-77 (0.5%)	100	100	85	85	50	66
Phytotoxin X-77 (1.0%)	99	100	95	92	50	63
Phytotoxin glycerin (1.0%)	25	85	50	35	3	32
Phytotoxin glycerin (3%)	23	100	73	84	32	35
Phytotoxin glycerin (3%) SYLGARD 309 (0.1%)	100	100	58	89	71	67
Phytotoxin SYLGARD 309 (0.1%)	100	100	60	100	98	90
Phytotoxin SYLGARD 309 (0.25%)	100	97	53	45	47	80
Phytotoxin TWEEN 20 (0.25%)	98	97	55	39	55	66
Phytotoxin TWEEN 20 (0.25%)	97	99	62	85	72	60

Phytotoxin was applied at a dose of 4.25 kg/ha.

% concentrations are expressed as v/v. Injury was evaluated 16 days after treatment

Table 10 Effects of Combinations of Toxin with Adjuvants

	% Injury							
Treatment	Velvet-leaf	Jimson-weed	Ragweed	Tomato	Johnson-grass	Barnyard-grass	Giant foxtail	Large Crabgrass
Water	0	0	0	0	0	0	0	0
Phytotoxin	40	76	45	24	7	0	40	42
Phytotoxin X-77 (0.5%)	79	99	50	65	99	75	75	90
Phytotoxin TRITON 100 (0.25%)	87	99	44	52	98	52	61	70
Phytotoxin Ammonium Sulfate (3 lb/A)	55	95	22	27	40	3	29	35
Phytotoxin AGRIDEX (.25%)	42	97	38	40	77	37	40	58
Phytotoxin CIDEKICK (.25%)	48	100	38	42	89	42	60	85
Phytotoxin DMSO (.25%)	50	85	61	58	76	0	47	40
Phytotoxin SILWET 7604 (.25%)	99	99	45	99	99	63	75	85
Phytotoxin SILWET 7614 (.25%)	95	91	37	64	91	75	70	65

Phytotoxin was applied at a dose of 4.25 kg/ha.

% concentrations are expressed as v/v. Injury was evaluated 14 days after treatment.

Table 11 Effects of Combinations of Toxin and Sulfosate

		% Injury					
Treatment	Rate	Velvet-leaf	Bind-weed	Morning-glory	Barnyard-grass	Giant foxtail	Johnson grass
Water	-	0	0	0	0	0	0
Phytotoxin	0.3 kg/ha	53	82	69	83	84	83
Phytotoxin	0.45 kg/ha	72	91	87	96	98	96
Phytotoxin	0.6 kg/ha	77	95	83	88	93	92

Sulfosate	0.28 kg/ha	40	67	40	64	96	77
Phytotoxin + Sulfosate	0.3 kg/ha 0.28 kg/ha	63	92	75	64	75	82
Phytotoxin + Sulfosate	0.45 kg/ha 0.28 kg/ha	87	97	80	91	98	90
Phytotoxin + Sulfosate	0.6 kg/ha 0.28 kg/ha	92	98	87	94	87	92

Sulfosate	0.56 kg/ha	40	40	42	79	95	95
Phytotoxin + Sulfosate	0.3 kg/ha 0.56 kg/ha	64	84	82	87	90	87
Phytotoxin + Sulfosate	0.45 kg/ha 0.56 kg/ha	83	97	91	86	87	92
Phytotoxin + Sulfosate	0.6 kg/ha 0.56 kg/ha	83	94	93	95	96	95

Table 11 (Continued)

Treatment	Rate	% Injury					
		Velvet-leaf	Bind-weed	Morning-glory	Barnyard-grass	Giant foxtail	Johnson grass
Sulfosate	0.84 kg/ha	47	45	40	70	91	95
Phytotoxin + Sulfosate	0.3 kg/ha 0.84 kg/ha	68	94	77	92	86	96
Phytotoxin + Sulfosate	0.45 kg/ha 0.84 kg/ha	74	87	74	91	95	96
Phytotoxin + Sulfosate	0.6 kg/ha 0.84 kg/ha	79	87	80	94	94	96

Response of 6 weed species to the toxin and tank mixes of the toxin and sulfosate 4 days after treatment showing earlier damage with the tank mix as compared to sulfosate alone. All treatments included 0.5% v/v X-77 as an adjuvant.

Table 12 Weed Species That Can Be Controlled by the Toxin

Broadleaf Weeds

Weed	Latin Bionomial
Bindweed, field	Convolvulus arvensis
Carpet Weed	Mollugo verticillata
Canada Thistle	Cirsium arvense
Chickweed	Stellaria media
Cocklebur	Xanthium strumarium
Dandelion	Taraxacum officinale
Hemp Sesbania	Sesbania exaltata
Henbit	Lamium purpureum
Jimsonweed	Datura stramonium
Lambsquarters	Chenopodium album
Leafy Spurge	Euphorbia esula
Morningglory	Ipomea sp.
Plantain, Buckhorn	Plantago lanceolata
Pigweed, Redroot	Amaranthus retroflexus
Sicklepod	Cassia obtusifolia
Ragweed, Common	Ambrosia artemisifolia
Velvetleaf	Abutilon theophrasti
White Clover	Trifolium repens

Table 12 (Continued)

Narrowleaf Weeds

Weed	Latin Bionomial
Barnyardgrass	Echinochloa crus-galli
Bermudagrass	Cynodon dactylon
Bluegrass, Annual	Poa annua
Bluegrass, Kentucky	Poa pratensis
Broadleaf Signalgrass	Bracharia platyphylla
Crabgrass, Large	Digitaria sanguinalis
Fall Panicum	Panicum dichotomiflorum
Foxtail, Giant	Setaria faberi
Foxtail, Green	Setaria viridis
Foxtail, Yellow	Setaria glauca
Goosegrass	Eleusine indica
Johnsongrass	Sorghum halepense
Johnsongrass, Rhizome	Sorghum halepense
Millet, Brown Top	Panicum ramosum
Quackgrass	Agropyron repens
Duckweed	Lemna minor

Table 13
Effects of Phytotoxin on Duckweed 5 Days After Treatment

Experiment 1

Toxin Rate (μg/mL)	% Injury
0.015	2
0.075	68
0.15	97
0.75	97
1.5	97

Experiment 2

Toxin Rate (μg/mL)	% Injury
0.03	7
0.075	75
0.12	95
0.15	97

Table 14 Detection of the Antibiotic Produced by Pseudomonas Syringae Strain ATCC #55090 with Test (Indicator) Strains

Indicator Strain	Producer Strain ATCC Strain #55090
Lactobacillus plantarum (ATCC 8014)	-
Micrococcus luteus (ATCC 13311)	-
Salmonella cholorasuis (ATCC 29213)	+
Pseudomonas aeruginosa (ATCC 27853)	+
Bacillus cereus (ATCC 11778)	+
Bacillus subtilis (ATCC 633)	+
Bacillus pumilus (ATCC 14884)	+
Bacillus subtilis (ATCC 9466)	B
E. coli (ATCC 25292)	+++

1 Indicator strains were suspended in a minimal media (PMS) which was allowed to solidify in plates. Subsequently, the producer strain was inoculated into the minimal medium continuing the indicator strain. Culture were incubated and colony diameter measured.

2 - = No activity

+ = Activity (1-9mm halo), ++ = (10-19mm halo)

+++ = (>20mm halo)

3 B = Borderline or trace activity

Table 15 Growth Inhibition of Bacterial Indicator Strains By a Semi-purified Antibiotic from Pseudomonas Syringae (Strain ATCC #55090)

Indicator Strain	Halo Diameter (mm) Toxin Dilution			
	10^0	10^{-1}	10^{-2}	10^{-3}
Pseudomonas andropogonis (ATCC 19311)	47.5	>30	28.7	19
Acetobacter diazotrophians (ATCC 49037)	36.4	25.7	ND	ND[1]
Pseudomonas aureofaciens (30-84)	41	22.6	14.6	8.6
Escherichia coli (ATCC 25922)	NT[2]	28	17.8	ND

[1] ND = Not Detected

[2] NT = Not Tested

Table 16 Pre-emergence Activity of TBL

Experiment 1

% Injury (19 Days After Treatment)

Treatment	Rate	Bluegrass	Barnyardgrass	Chickweed	Tomato
Control (X-77 + Water)	0.25%	0	0	0	0
Phytotoxin 99	3.2 kg/ha	90	65	64	
Phytotoxin 100	6.4 kg/ha	100	98	87	
Phytotoxin 99 + X-77	3.2 kg/ha	99	70	75	
Phytotoxin 100 + X-77	6.4 kg/ha	99	86	91	

Experiment 2

% Injury (16 Days after Treatment)

Treatment	Rate	Fall Panicum	Barnyardgrass	Morningglory	Tomato
Control + X-77 0	0.25	0	0	0	
Phytotoxin 68 + X-77	1.6 kg/ha	89	62	64	
Phytotoxin 88 + X-77	3.2 kg/ha	97	68	63	

X-77 was included at a rate of 25% (v/v).

Table 17 Comparison of Herbicides and Phytotoxin in Duckweed Bioassay

Compound	Well Concentration (μM) 100% Control	50% Control
Phytotoxin	0.74	0.22
L-Methionine sulfoximine	5	2
Glufosinate	25	12
Bialaphos	7	5
Glyphosate	25	10
Paraquat	5	1

WHAT IS CLAIMED IS:

1. A herbicidal composition comprising tabtoxinine-β-lactam or a derivative or salt thereof.

2. The herbicidal composition of claim 1, wherein said derivative is tabtoxin.

3. The herbicidal composition of claim 1, wherein said tabtoxinine-β-lactam is produced by Pseudomonas syringae (ATCC #55090).

4. The herbicidal composition of claim 1, further comprising an adjuvant, wherein said adjuvant is a nonionic surfactant.

5. The herbicidal composition of claim 4, wherein said nonionic surfactant is a neutral organosilicone surfactant.

6. The herbicidal composition of claim 1, further comprising sulfosate.

7. The herbicidal composition of claim 1, further comprising glyphosate.

8. A process of controlling weeds comprising topically applying tabtoxinine-β-lactam or a derivative or salt thereof to said weeds.

9. The process of claim 8, wherein said tabtoxinine-β-lactam is sprayed onto said weeds.

10. The process of claim 8, wherein said derivative is tabtoxin.

11. The process of claim 10, comprising the steps of combining tabtoxin with adjuvants, and applying said tabtoxin to weeds.

12. The process of claim 8, wherein said toxin is produced by Pseudomonas syringae (ATCC #55090).

13. The process of claim 8, comprising the steps of combining tabtoxinine-β-lactam or a derivative or salt thereof with adjuvants, and applying said tabtoxinine-β-lactam or derivative or salt thereof to weeds.

14. The process of claim 13, wherein said adjuvant is ORTHO-X77®.

15. The process of claim 8, wherein said tabtoxinine-β-lactam is dissolved in a solution having an acid pH.

16. A process for the production of tabtoxinine-β-lactam comprising the steps of cultivating Pseudomonas syringae (ATCC #55090) bacteria or mutants or variants thereof;

separating said bacteria from the culture medium;

and isolating said tabtoxinine-β-lactam.

17. The process of claim 16, wherein said isolating comprises binding said tabtoxinine-β-lactam to an ion exchange material and eluting said tabtoxinine-β-lactam from said ion exchange material.

18. The process of claim 17, wherein said ion exchange material is a cation exchange material.

19. The process of claim 16, wherein said isolating comprises binding said tabtoxinine-β-lactam to activated carbon and eluting said tabtoxinine-β-lactam from said activated carbon.

20. A process of controlling weeds comprising applying tabtoxinine-β-lactam or a derivative or salt thereof to soil.

21. The process of claim 20, wherein said derivative is tabtoxin.

22. The process of claim 20, wherein said toxin is produced by Pseudomonas syringae (ATCC #55090).

23. Tabtoxinine-β-lactam produced by the process comprising the steps of

culturing Pseudomonas syringae ATCC #55090;

removing the live bacteria; and

isolating tabtoxinine-β-lactam from the culture medium.

24. A herbicidal composition comprising tabtoxinine-β-lactam produced by the process claimed in claim 23.

25. A method of defoliating plants comprising treating said crop plants with tabtoxinine-β-lactam or a derivative or salt thereof.

26. The process of claim 25, wherein said derivative is tabtoxin.